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13. ABSTRACT *(Maximum 200 Words)*

The ability of a tumor cell to grow outside its local environment (metastasize) is a major problem in the development and therapeutic treatment of breast cancer. The loss of the requirement for cell-matrix interactions plays a critical role in the development of cancer. Normal epithelial cells require attachment to the extracellular matrix (ECM) not only for proliferation but also for survival, as disruption of cell-ECM interactions can result in reversible cell growth arrest and induction of apoptosis, a phenomenon termed "anoikis". Elucidating the mechanisms by which the interactions of cells with the ECM generate and regulate downstream signals that promote cell growth and survival is critical to understanding cancer. Activation of actomyosin contractility is an essential step during adhesion-dependent signaling. We are studying the role of specific components of the actin cytoskeleton whose expression has been implicated in the transformed phenotype. These studies will provide important new information concerning the role of these actin filament-associated proteins in adhesion-dependent cell signaling and the potential of inhibiting signal transduction pathways dependent on actomyosin contractility as a therapeutic target and adjuvant for the treatment of breast cancer, as well as other cancers.

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breast cancer, epithelial cells, extracellular matrix (ECM), apoptosis, anoikis, actomyosin, cytoskeleton, actin, tropomyosin, caldesmon

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INTRODUCTION:

The goal of this research program is to contribute to our understanding of the molecular basis of metastasis. The ability of a tumor cell to grow outside its local environment (metastasize) is a major problem in the development and therapeutic treatment of cancer. Adhesion of cells to the extracellular matrix (ECM) and neighboring cells plays a critical role in various cellular processes linked to transformation including differentiation, growth, motility and programmed cell death (apoptosis). Regulation of cell death is an essential component in the body's defense against the emergence of cancer. Attachment of cells to the ECM is important for the generation of signals that regulate normal cell proliferation and apoptosis. The loss of the requirement for cell-matrix interactions plays a critical role in the development of cancer. Recent experiments from our and other laboratories have shown that activation of actomyosin contractility is an essential step during adhesion-mediated signal transduction. In addition, this suggests a mechanism by which changes in cellular proteins involved in the regulation of myosin function lead to aberrant growth control by constitutively activating downstream signaling pathways. The experiments outlined in this grant will test the hypothesis that constitutive activation of actomyosin contractility contributes, in part, to the transformed phenotype and to the inability of transformed cells to undergo apoptosis. Specifically, we will determine if inhibition of myosin by pharmacological agents or overexpression of caldesmon and tropomyosin (TM), two cellular proteins that can regulate myosin function, results in activation of the apoptotic pathway in transformed cells. In addition, studies from our laboratory show that TM plays a causal role in the phenotype of ras-transformed fibroblasts (Gimona et al., 1996), and we have shown for the first time that nonmuscle caldesmon plays a key role in the regulation of actomyosin contractility and adhesion-mediated signaling in nonmuscle cells (Helfman et al., 1999). The experiments outlined in this grant will determine whether changes in TM and caldesmon in epithelial cells contribute to the transformed phenotype, as has been demonstrated for fibroblasts. We suggest that TM and caldesmon function as part of a cellular feedback mechanism to regulate various signal transduction pathways dependent on actomyosin contractility. Our studies will provide important new information on the role of these proteins in adhesion-dependent cell signaling and the potential of inhibiting signal transduction pathways dependent on actomyosin contractility as a therapeutic target and adjuvant for the treatment of breast cancer, as well as other cancers.

BODY:

The observation that activation of actomyosin contractility is an essential step during adhesion-mediated signal transduction suggests a mechanism by which changes in cellular proteins involved in the regulation of myosin function in transformed cells will contribute to aberrant growth control. The experiments outlined for this grant in the original "**Statement of Work**" included experiments to: (1) analyze the effects of treating cells with pharmacological agents to determine if inhibition of actomyosin contractility induces apoptosis and (2) test transient transfection of caldesmon on apoptosis. These experiments were designed to test the hypothesis that constitutive activation of actomyosin contractility contributes, in part, to the transformed phenotype and to the insensitivity of transformed cells to undergo apoptosis. Accordingly, during this funding period we determined if inhibition of myosin results in activation of the apoptotic pathway in adhesion-independent cells. If activation of actomyosin contractility is a critical step in the pathway of adhesion-dependent suppression of apoptosis, then inhibiting myosin function will promote apoptosis. These studies provided important new information concerning the role of actin filaments and myosin in adhesion-dependent cell signaling and the potential of inhibiting signal transduction pathways dependent on actomyosin contractility as a therapeutic target and adjuvant for the treatment of breast cancer, as well as other cancers.

KEY RESEARCH ACCOMPLISHMENTS:

The experiments conducted during the current funding period were designed to determine if activation of actomyosin contractility plays a role in intracellular signaling following cell-ECM interactions and thereby prevents apoptosis. Accordingly, if loss of cell-ECM interactions results in a decrease in actomyosin contractility and thereby activates the apoptotic pathway in normal cells, then inhibition of myosin itself should lead to apoptosis. In addition, if transformed cells are able to exhibit adhesion-independent cell growth because of constitutive activation of actomyosin contractility, then inhibiting myosin II function should effect cell growth and cell survival. Accordingly, we studied the effects of altering myosin II function in normal MDCK cells and a variety of transformed human mammary epithelial cells. The normal epithelial cell line exhibits the same pattern of TM expression as primary epithelial. By contrast, the various ductal and adenocarcinoma cell lines exhibit a loss of specific high molecular weight TMs already implicated in transformation of fibroblasts. These cells provide a useful model to study the relationships between changes in TM expression and cell transformation for experiments outlined in Specific Aims 2 and 3 of this grant.

We used two different compounds that inhibit myosin II, namely Butanedione monoxime (BDM) and ML-7. BDM inhibits the ATPase activity of myosin and ML-7 blocks myosin light chain kinase and thereby inhibits myosin II contractility. Apoptosis was be assayed by staining cells with DAPI and viewing the nuclei or by analysis of DNA degradation. We also assayed for other known markers of apoptosis including staining cells for annexin V, and processing of caspases. For the studies we continually obtain the advice of Drs. Michael Hengartner, Yuri Lazebnick, Scott Lowe, at Cold Spring Harbor Laboratory, who are experts in apoptosis. We treated cells with 2,3-butanemonoxime (BDM) and ML7 (Zhong et al., 1997), both of which have been shown to inhibit the function of myosin II contractility. BDM inhibits the release of ATP from the myosin head, and therefore inhibits binding of myosin to actin, and ML7 inhibits MLCK. Latrunculin A and cytochalasin D were used to determine the role of actin inhibition on cell death. Latrunculin A binds to actin monomers and prevents polymerization of actin. Cytochalasin D binds to actin filaments preventing association or dissociation from that end (Brown and Spudich, 1981). We have also used the general caspase inhibitor zVAD-FMK to inhibit activation of caspases in treated cells. Cells treated with this compound should resemble untreated cells i.e, no caspase activation. Cells were treated for various time points and concentrations to determine desired amount of cell death.

Activation of caspase 8 in cells treated with myosin inhibitors:

MDCK, MDCK Ras, and MCF7 cells were treated with BDM or ML7 to determine the importance of myosin activation in these cells. They were tested for the presence of an activated caspase. Activated caspases are cleaved and of smaller molecular weight than the inactive form. Activation of caspase-8 was examined because this is an initiator caspase. An overnight treatment of cells with 50mM BDM was harvested, a whole cell extract was prepared, and extracts were run on a 10% SDS gel. This was transferred to nitrocellulose, and blots were probed with a caspase-8 monoclonal antibody, which recognizes inactive caspase-8 (55kD) and active caspase–8 (45kD). Figure 1 shows the activation of caspase-8 with myosin inhibition in MDCK and MDCK Ras cells. zVAD-FMK, a general caspase inhibitor, was used to test for inhibition of caspase-8 activation in treated cells. Figure 1a clearly shows the disappearance of the activated caspase-8 with zVAD-FMK. Figure 1b shows similar results of myosin inhibition with BDM in MDCK Ras cells. Figure 1c shows MDCK cells treated with another myosin inhibitor, ML7. Activated caspase-8 is again shown with an overnight treatment at 30um and 40um. Again, disappearance of the band with zVAD-FMK is seen. Similar results were found with activation of caspase-8 in MCF7 cells (data not shown).

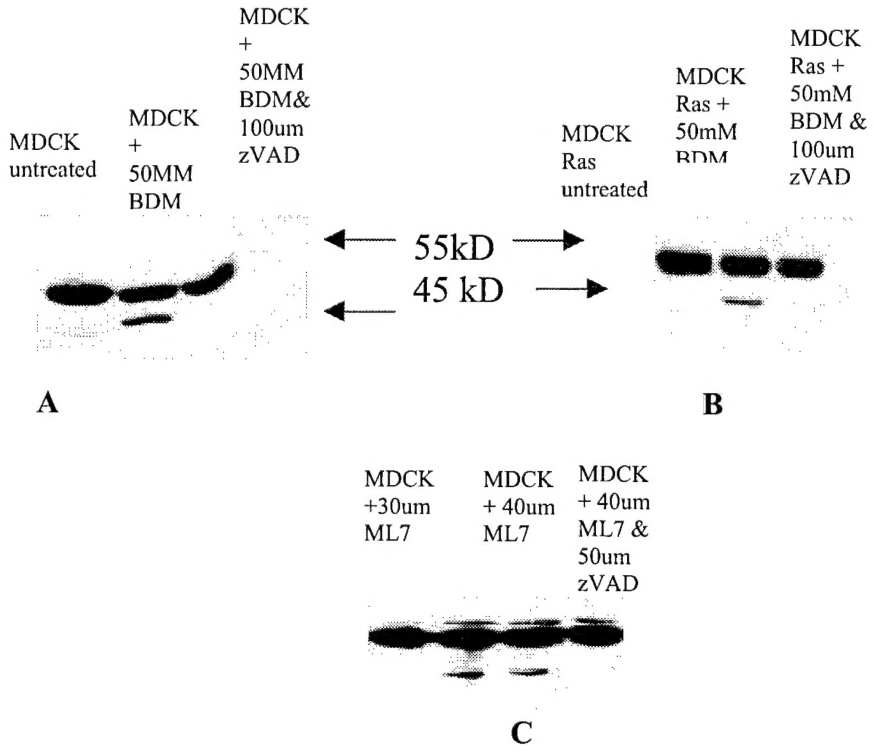

Figure 1: MDCK (a) and MDCK Ras (b) cells treated with 50mM BDM and 50mM BDM + zVAD-FMK overnight and c) MDCK cells treated with 30um , 40um ML7 and 40um ML7 +zVAD-FMK overnight. Blots were probed with anti caspase-8 monoclonal antibody. The activation of caspase-8 is indicated by the appearance of a 45kD band.

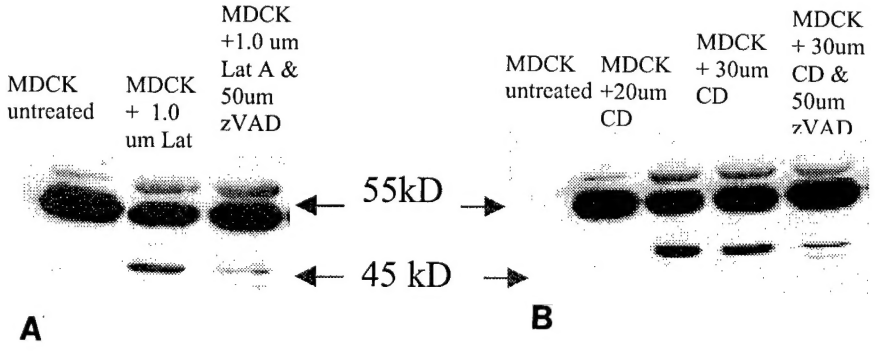

Figure 2: MDCK cells treated with latrunculin A (A) and cytochalasin D (B) with the indicated concentrations overnight. Blots were probed with anti caspase-8 antibody, and activated caspase-8 is seen with treatment of the actin disrupting drugs.

Cells were also treated with latrunculin A, a compound that binds to actin monomers preventing polymerization, and cytochalasin D, a compound that binds to the barbed ends of actin filaments, preventing further polymerization. After an overnight treatment of MDCK cells with either latrunculin A (Fig. 2a) or cytochalasin D (Fig.2b), cells were extracted, run on a 10% gel and transferred to a nitrocellulose membrane. The blots were probed with anti caspase-8 antibody to determine induction of apoptosis. With both latrunculin A and cytochalasin D caspase-8 is activated, and activation is inhibited with zVAD-FMK. These results demonstrate the induction of apoptosis in cells where actomyosin contractility is inhibited.

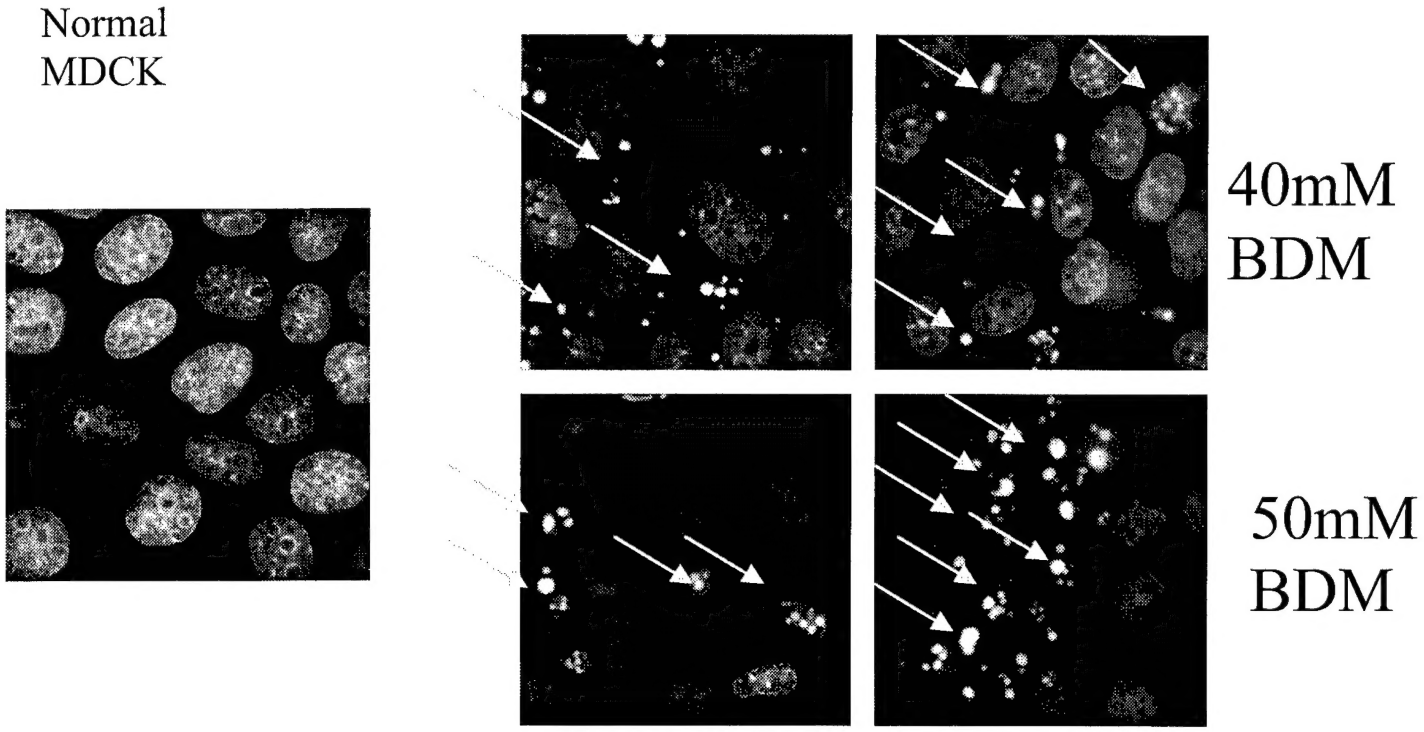

Figure 3: MDCK cells treated with BDM for 24 and 48h. Arrows indicate chromatin condensation of apoptotic cells.

FACS analysis of cells treated with myosin and actin inhibitors

Apoptosis is characterized by many morphological changes, including chromatin condensation, cell shrinkage, and membrane blebbing (Thornberry and Lazebnik, 1998). To confirm apoptosis in cells treated with inhibitors of myosin or actin, MDCK cells were grown on coverslips, treated with varying concentrations of BDM after 24 or 48 hours, then fixed with 3% paraformaldehyde and permeablized with 0.1% Triton. The cells were then stained with DAPI, which binds to cell DNA. Examination of the coverslips on a fluorescent microscope revealed the presence of many condensed nuclei (Figure 3). Similar results were found with treatment of MDCK cells with ML7, latrunculin A and cytochalasin D (data not shown).

Table I below summarizes the data from our analyses. As indicated in Table I, when normal MDCK cells were plated on poly-HEMA approximately 50% of the cells underwent programmed cells death, in agreement with previous studies showing these cells undego apoptosis when deprived of the substratum (Frisch et al., 1994). By contrast the human breast cancer cell line MCF7 was relatively resistant to loss of matrix interactions. However, treatment of normal or transformed cells with inhibitors of myosin or agents that disrupt the actin cytoskeleton resulted in induction of apoptosis. For example, treatment of the MCF7 cells with BDM or ML7 resulted in apoptosis in 60-70% of the cells. These results are in agreement with our hypothesis that activation of actomyosin contractility is a critical step in the generation of signals that prevent apoptosis. In addition, the same results were obtained using two different agents to inhibit myosin, namely BDM and ML7 that have different mechanisms of actin to inhibit myosin II activity. Butanedione monoxime (BDM) inhibits the ATPase activity of myosin, and ML7 blocks myosin light chain kinase and thereby inhibits myosin II contractility. In addition, disruption of the cytoskeleton by latrunculin A or cytochalasin D also resulted in a larger percentage of cells undergoing apoptosis compared to cells plated on polyHEMA. The induction of apoptosis by the various pharmacological agents was not simply due to disruption of adhesion of cells to the substratum, because identical results were obtained when cells were analyzed following fixation and staining for DAPI to score cells for chromosome condenstation (see Figure 3 above).

Table 1: % Living Cells After Overnight Treatment

	Normal Untreated	Polyhema	40mM BDM	30uM CD	1.0 um Lat A	40uM ML7
MDCK	85	52	45	52	43	36
MDCK Ras	95	94	58	75	50	58
MCF7	92	71	62	-	-	54
DU145	91	76	53	-	-	47

In agreement with our hypothesis, inhibition of actomyosin contractility causes apoptosis in both normal and transformed epithelial cells. Using inhibitors of myosin and actin function, we have demonstrated that normal and transformed epithelial cells will undergo apoptosis. This is demonstrated with the presence of activated caspases, is shown clearly by the presence of chromatin condensation, and by staining cells for apoptotic markers.

The second method we are using to investigate the role of cell contractility in adhesion-dependent signaling and apoptosis is to study the effects of caldesmon. We have recently found that overexpression of nonmuscle caldesmon in fibroblasts blocks cell contractility (Helfman et al., 1999). Thus caldesmon is a potent and specific tool that can regulate myosin-II contractility. We are using transient transfection of epitope-tagged constructs into MDCK cells and various human mammary epithelial cells. Cells are fixed and stained at 24, 48 and 72 hours post-transfection. and analyzed by immunostaining of cells transfected with tagged-isoforms and cells stained for markers to assess the apoptotic response.

The results of our data indicate that agents that inhibit myosin II function lead to programed cell death in epithelial cells. Thus, actomyosin contractility plays a critical role in the generation of signals required for the prevention of apoptosis in epithelial cells (Pawlak and Helfman, 2001 [Appendix]).

REPORTABLE OUTCOMES:

This funding provides research support for a doctoral student and a postdoctoral fellow.

CONCLUSIONS:

The results of our studies are in agreement of our hypothesis concerning the role myosin II function plays a critical role in adhesion-dependent cell growth and in the apoptotic response of cells. In this regard it is interesting to note that Rho plays a role in both transformation (Qiu et al., 1995) and metastasis (Yoshioka et al, 1998). Rho is a downstream effector of the ras pathway is known to stimulate the actomyosin system and this is thought to play a direct role in invasion of tumor cells (Yoshioka et al., 1998). While this role is believed to involve increase motility of tumor cells, it is also possible that activation of contractility alternatively activates signal transduction pathways associated with adhesion-dependent cell growth, that would antagonize the normal apoptotic pathway and lead to survival of tumor cells outside their normal environment. Thus our studies have provided important new information on the role of actomyosin contractility in adhesion-dependent cell signaling and the potential of inhibiting signal transduction pathways dependent on actomyosin contractility as a therapeutic target and adjuvant for the treatment of breast cancer, as well as other cancers.

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APPENDIX:

Cytoskeletal changes in cell transformation and tumorigenesis. Geraldine Pawlak and David M. Helfman. *Current Opinion in Genetics & Development* 2001, 11:41-47.

Cytoskeletal changes in cell transformation and tumorigenesis

Geraldine Pawlak* and David M Helfman†

Research during the past couple of years has provided important new information as to how the actin cytoskeleton contributes to growth control in both normal and transformed cells. The cytoskeleton can no longer be viewed as simply a structural framework playing a role in cell shape and motile events such as cell movement, intracellular transport, contractile-ring formation and chromosome movement. More recent experiments show that the cytoskeleton plays a critical role in the regulation of various cellular processes linked to transformation including proliferation, contact inhibition, anchorage-independent cell growth, and apoptosis.

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Abbreviations

ECM	extracellular matrix
ERM	ezrin/radixin/moesin
GEF	guanine nucleotide exchange factor
MAPK	mitogen-activated protein kinase
MLC	myosin light chain
PAK	p21-activated kinase
ROCK	Rho kinase
Tiam1	T-lymphoma invasion and metastasis 1
TM	tropomyosin

Introduction

Transformation of cells in tissue culture results in a variety of cellular changes including alterations in serum- and adhesion-dependent cell growth, loss of contact inhibition, changes in adhesiveness, motility, morphology, and organization of the cytoskeleton. Disruption of actin filaments and a decrease in focal adhesions are common features following transformation of cells by various oncogenes. These changes in microfilament structure are highly related to both anchorage-independent growth and cellular tumorigenicity, suggesting fundamental roles for actin filaments in oncogenic transformation. The alterations in actin filament structure were found to correlate with decreased expression of various cytoskeleton proteins [1]. Subsequent experiments were carried out in order to determine if the decrease in the expression of specific actin filament-associated proteins contribute to the transformed phenotype. These studies demonstrated that the re-expression of these proteins, via transfection, frequently suppresses many features of transformation including restored formation of microfilament bundles, focal adhesions, contact-inhibited cell growth, inability to grow on soft agar, and suppression of tumorigenicity in nude mice. These studies include the actin-associated proteins α-actinin [2,3], gelsolin [4], profilin [5], vinculin [6] and tropomyosin (TM) [7,8•,9–12]. Although the mechanisms by which these proteins contribute to growth control remain to be fully elucidated, these studies demonstrate that changes in the expression of specific structural components of the actin cytoskeleton can contribute to transformation. In addition, much effort has been directed towards understanding how the Ras/Rho family of GTPases (Rho, rac and cdc42) regulates actin-filament assemblies in both normal and transformed cells [13,14•,15,16,17•]. Here, we review recent studies that shed light on how structural components of the actin cytoskeleton contribute to changes in microfilament assembly and how changes in these proteins might contribute to alterations in growth control and tumorigenesis.

Actin-filament assemblies, small GTPases and transformation

Oncogenic activity of small GTPases can target actin-containing structures by several mechanisms including alterations in the expression of cytoskeletal proteins (e.g. TM) or interaction with cytoskeletal proteins (e.g. ezrin and ankyrin). Studies of Ras-transformed cells have raised some intriguing issues regarding the actin cytoskeleton and the role of Rho family proteins in transformation. Rho is thought to act downstream of Ras but whereas Rho activation is known to promote the development of stress fibers and focal adhesions, Ras-transformed fibroblasts often exhibit a loss of these structural elements. Interestingly, activated RhoA can restore stress fibers in Ras-transformed cells [18]. Conversely, inhibition of Rho kinase (ROCK) — an effector of RhoA that is involved in stress-fiber formation — can block Ras transformation [19]. One possibility is that the frequently observed downregulation in the expression of various actin filament associated proteins is required for transformation by Ras and perhaps other oncogenes. The TMs are a family of rod-shaped proteins that bind along both grooves of filamentous actin [20]. TMs bound along actin filaments can stabilize filaments by protecting them from the action of severing proteins. Decreased expression of high molecular weight TM isoforms (284 amino acids) is found in fibroblasts transformed by various oncogenes, carcinogens, DNA and RNA tumor viruses [20]. In addition to studies using cells in culture, changes in TM expression have been found in human prostate cancer and breast cancer [21–23]. More recent studies [24•–26•] using microarrays have reported changes in TM expression and other components of the actin cytoskeleton in metastatic melanoma cells and MYC-transformed cells. At present, the mechanism for downregulation of TM expression in *ras*-transformed cells is not known. In one study, the *ras*-induced downregulation of TM was found to be Raf-mediated, but

MEK-independent because treatment with the MEK1 inhibitor PD98059 had little effect on TM levels, suggesting that a novel pathway exists downstream of Raf which may play an important role in the regulation of the cytoskeleton [27]. In a study of c-Jun transformed cells, however, the downregulation of TM was reversed following treatment of cells with the MEK1 inhibitor PD98059 [28]. Clearly more work will be required to understand the pathways leading to the decreased synthesis of TM. Properties of Ras-transformed and *src*-transformed cells were reverted to normal following forced expression of specific high molecular weight TMs, including restored formation of microfilament bundles, contact-inhibited cell growth, inability to grow on soft agar, and suppression of tumorigenicity in nude mice. [7,8•,10,12]. How forced expression of TM can lead to suppression of the transformed phenotype is still not understood. In addition to a role in stabilizing actin filaments, TM might play a role in the regulation of myosin II function and adhesion-dependent signaling (see below).

A role for ezrin/radixin/moesin (ERM) proteins in transformation has been reported [29••,30••]. Early studies implicated ERM proteins in transformation as they were found to be overexpressed upon transformation [31,32] and in metastatic cell lines [33,34]. ERM proteins form crosslinks between cortical actin filaments and the plasma membrane. They play a role in formation of microvilli, cell-adhesion sites, lamellipodia and contractile rings during cytokinesis. ERM proteins are also structurally related to the tumor-suppressor protein merlin/schwannomin, which is involved in neurofibromatosis type II [35•]. Overexpression of merlin/schwannomin in 3T3 cells suppresses the Ras-transformed phenotype (i.e. anchorage-independent growth on soft agar and restores contact inhibition of cell growth) [36,37]. Recently, the TSC1 tumor-suppressor hamartin was found to interact directly with ERM proteins [29••]. The interaction of hamartin with ERM proteins is required for the activation of Rho by serum or lysophosphatidic acid, which normally results in the assembly of actin stress fibers and formation of focal adhesions. The loss of hamartin is thought to result in disruption of cell adhesion to the extracellular matrix (ECM), which may initiate the development of TSC hamartomas. These studies also indicate that a Rho-mediated signaling pathway regulating cell adhesion may constitute a rate-limiting step in tumor formation. Whether hamartin activates Rho through the activation of a guanine nucleotide exchange factor (GEF) or by suppression of the activity of a guanine nucleotide dissociation inhibitor remains to be determined. In a related study ezrin was found to be required for ROCK-mediated transformation by the Net and Dbl oncogenes which are RhoA GEFs [30••]. ROCK was found to phosphorylate ezrin on threonine 567 and mutation of this site to alanine inhibited ROCK-induced relocalization of ezrin to actin-containing structures, and also inhibited RhoA-mediated contractility and focal-adhesion formation. Interestingly, ezrin T567A (but not wild-type ezrin) specifically inhibited transformation by the RhoGEFs Dbl and Net but not by v-*src*, suggesting that ezrin activation is an essential target in transformation induced by these oncogenes. The ezrin T567A also inhibited *ras*-transformation, in agreement with the observation that RhoA-signaling pathways contribute to transformation by Ras [19,38]. This study shows that ezrin-mediated cytoskeletal rearrangements induced by ROCK are required for RhoA- and RhoGEF-induced transformation.

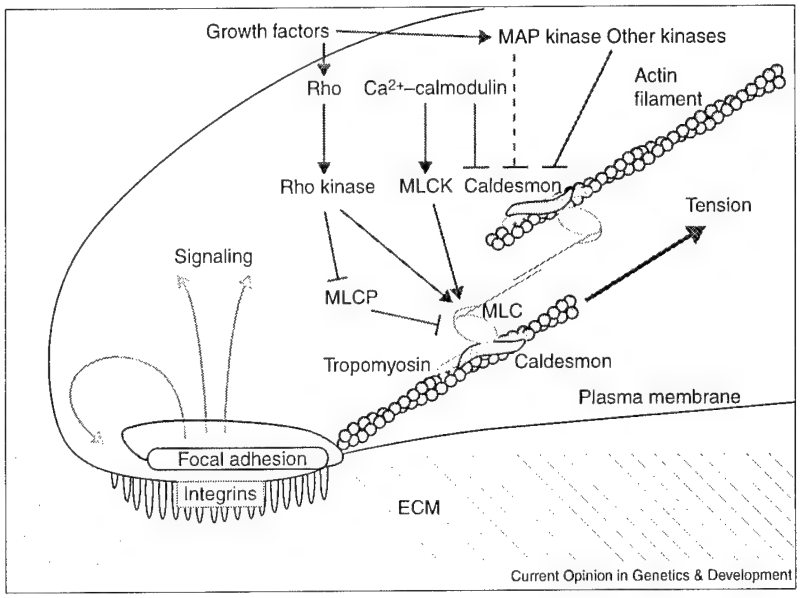
Tiam1 (T-lymphoma invasion and metastasis 1) is one of the GEFs for Rac1, whose expression was implicated in metastasis [39]. Recent studies of the mechanism of Tiam1–Rac1 signaling in metastatic breast tumor cells reveal that Tiam1 interacts with ankyrin [40••]. Ankyrin belongs to a family of cytoskeletal proteins that couple a variety of membrane-spanning cell surface proteins to the spectrin–actin cytoskeleton on the cytoplasmic surface of the plasma membrane [41•]. Importantly, the binding of Tiam1 to ankyrin activates the GDP/GTP exchange on Rac1. This study suggests that the interaction of Tiam1 with ankyrin plays a critical role in regulating Rac1-activated oncogenic signaling and cytoskeleton-mediated metastatic breast tumor cell progression.

Actomyosin contractility in adhesion-dependent signal transduction

Cell adhesion involves dynamic interactions between the ECM, various transmembrane adhesion receptors, and the actin cytoskeleton. These interactions play a critical role in the regulation of cell growth, differentiation and survival, as well as cell shape and motility. During tumor cell progression a number of cellular features are altered including acquisition of unregulated proliferation and the ability of cells to grow outside their local environment (metastasize)-the latter feature being a major factor in the development of cancer and a problem in its treatment. The loss of adhesion-responsive regulation of cell growth is a characteristic feature that distinguishes transformed cells from normal cells. Cell growth often becomes independent of growth factors and adhesion (anchorage-independent) in transformed cells, as exemplified by their ability to grow on soft agar.

Adhesion of cells to the ECM via transmembrane receptors of the integrin family induces a rapid sequence of events including structural alterations (formation of focal contacts and microfilament bundles), as well as signal transduction (e.g. tyrosine phosphorylation of focal adhesion kinase [FAK], paxillin and p130cas), ultimately resulting in regulation of the cell-cycle machinery [42,43••]. Critical cellular components involved in this adhesion-dependent signaling are specialized structures called focal adhesions. At these sites, clustered integrins span the plasma membrane and interact extracellularly with components of the ECM, and on the cytoplasmic side with cytoskeletal proteins that function in the attachment of bundles of actin filaments (stress fibers) to these regions. Proteins involved in signal transduction are also concentrated at these sites. Although certain steps in the signaling cascade are well characterized,

Figure 1



Proposed model for the involvement of actin filaments, myosin II, TM and caldesmon in the regulation of cell contractility and adhesion-dependent signaling. Actomyosin contractility in non-muscle cells involves a number of structural components including actin, myosin II, TM, and caldesmon. Tension on integrins at focal adhesions developed by the actin system affects adhesion-dependent signaling. This signaling, in turn, promotes further assembly of the focal adhesions and also induces downstream events such as cell proliferation and suppression of apoptosis. Tension is positively regulated by MLC kinase (MLCK) and Rho via Rho-kinase and negatively regulated by caldesmon. Caldesmon is potentially regulated by a number of kinases (see main text for discussion). Inhibition of the actin-activated myosin ATPase activity by caldesmon is dependent on TM. For simplicity, isolated copies of TM and caldesmon are shown, though in fact they are localized along the actin filaments. MLCP, MLC phosphatase.

the complete mechanism of adhesion-dependent signaling is not fully understood. In addition to attachment to the appropriate ECM, the activation of adhesion-dependent signaling requires co-stimulation by soluble ligands (growth factors), whose effects are mediated by the small GTP-binding protein Rho [13,44]. One target of Rho is activation of ROCK, which phosphorylates, and thereby inactivates myosin light chain (MLC) phosphatase resulting in activation of myosin contractility [44,45]. Thus, activation of myosin II contractility is a target during adhesion-dependent signaling.

Recent studies have demonstrated that activation of myosin II (i.e. actomyosin contractility), is a critical step in adhesion-dependent signaling via its effects on formation of focal adhesions [46–48,49••]. Pharmacological agents that block actomyosin contractility inhibit Rho-induced formation of stress fibers and focal adhesions, as well as phosphorylation of FAK and paxillin [47]. More recent results [49••] show that overexpression of caldesmon, a protein involved in inhibition of actomyosin contractility, blocks Rho-induced formation of stress fibers and tyrosine phosphorylation of focal adhesions. The mechanism by which increased contractility results in formation of stress fibers, focal adhesions and elevated tyrosine phosphorylation is not fully understood. One hypothesis is that integrins are normally loosely associated within the cell membrane but stimulation of myosin contractility results in bundling of the actin, generating tension that would aggregate the integrins [47,50]. Integrin clustering activates FAK, leading to FAK autophosphorylation and recruitment to the developing focal adhesion of various signaling proteins, which would account for the increase in tyrosine phosphorylation observed in Rho-stimulated cells [51,52]. These studies demonstrate that the actin cytoskeleton plays a key role in cell signaling.

Most studies of the regulation of nonmuscle contractility and its relationship to adhesion-dependent signaling have focused on the role of MLC phosphorylation and its upstream regulators. The observation that activation of actomyosin contractility is an essential step for adhesion-dependent signal transduction suggests a mechanism by which changes in actin-filament-associated proteins could contribute to aberrant growth control (Figure 1). In principle, changes in the levels of cellular proteins involved in the regulation of myosin function observed in transformed cells could lead to activation of actomyosin contractility and contribute to activation of the downstream signaling pathways that are required for cell growth. TM is a natural partner of caldesmon in its regulation of actomyosin-based contractility. *In vitro* studies [53] have demonstrated differences in the abilities of smooth muscle and skeletal muscle TMs to act cooperatively with caldesmon to inhibit myosin II function. On the basis of the results demonstrating an ability of caldesmon to regulate adhesion-dependent signaling [49••], interaction of caldesmon with specific TMs may be important in maintaining the normal signaling functions in the cell. How different nonmuscle TMs will effect the actions of caldesmon on myosin is not known. A loss of specific TMs could contribute to activation of adhesion-dependent signaling pathways as a result of unregulated actomyosin contractility. Caldesmon has been reported to be phosphorylated by various kinases including cdc2, casein kinase II, protein kinase C, cyclic AMP-dependent protein kinase, calcium/calmodulin kinase, mitogen-activated protein kinase (MAPK) and p21-activated kinase (PAK) [54–59].

Phosphorylation of caldesmon may be a target for the regulation of signaling pathways. Studies of caldesmon in smooth muscle have shown it to be phosphorylated by PAK and this phosphorylation was correlated with increased actomyosin contractility [59], although it is as yet unknown if PAK plays a role in the regulation of caldesmon function in nonmuscle cells. In addition, PAK has been implicated in microfilament reorganization and invasiveness of breast-cancer cells [60]. Another line of evidence for caldesmon in signaling and transformation has been suggested in studies showing that caldesmon is tyrosine phosphorylated in a complex with Shc–Grb2–Sos in v-ErbB-transformed cells [61,62,63••]. Clearly, further studies will be required to determine the role played by TM and caldesmon in nonmuscle myosin function and intracellular signaling.

Apoptosis

The role of the actin cytoskeleton in the execution phase of apoptosis — characterized by morphological changes such as cell rounding, membrane blebbing, and chromatin condensation — is well established [64•]. However, the cytoskeleton can also play a role in the regulation of apoptosis as suggested by the finding that cytoskeletal disruption by cytochalasin D can induce apoptosis [65]. In addition, apoptosis driven by various signals is accompanied by the specific cleavage of actin-associated proteins, including gelsolin [66], fodrin [67], adducin [68] and myosin heavy chain [69]. These studies suggest that disruption of the cytoskeleton is a required step during apoptosis. Modification of the actin cytoskeleton state also impacts upon signals leading to apoptosis: stabilization of actin filaments by jasplakinolide was shown to enhance apoptosis induced by cytokine deprivation [70]. Integrity of the cytoskeleton has been shown to be essential for CD95-induced apoptosis, through an ezrin-mediated association between CD95 and the actin cytoskeleton [71••]. Conversely, cytoskeleton disruption by cytochalasin D accelerates DNA-damage-induced apoptosis [72]. The apparent discrepancies between these studies may reflect cell-type specificity. Much of our understanding of cytoskeleton function is derived from studies of fibroblast cell lines and it is conceivable that the cytoskeleton plays different roles in signal transduction events depending on the cell type. Nonetheless, the actin cytoskeleton appears to play a critical role in the regulation of cell response to apoptotic signals but how it regulates downstream targets remains to be determined.

Disruption of cell–ECM interaction results in a reversible induction of apoptosis — a phenomenon termed 'anoikis' [73–75]. This process is believed to play a critical role in preventing the growth of cells outside their local environment (i.e. metastasis). Conversely, inhibition of anoikis in tumor cells allows them to invade surrounding tissues. Several molecules and signaling pathways induced by interaction of the cell with the ECM have been implicated in the regulation of anoikis [43••], among them FAK, phosphatidylinositol-3-kinase, Akt, nuclear factor (NF)-κB, and MAP kinases. Activation of FAK is a critical component as it prevents anoikis [76]. The downstream effectors remain to be established but recent studies indicate that FAK can suppress p53-mediated apoptosis [77]. Furthermore, p53 serves to monitor survival signals from the ECM/FAK as apoptosis is suppressed by dominant negative p53. In addition to FAK, a second component of focal adhesions, namely CAS, has been implicated in regulation of apoptosis [78,79••]. The CAS proteins — p130Cas, HEF1/Cas-L and Efs/Sin — are a family of docking proteins that contain multiple interaction domains, and are important components of integrin-receptor signaling [80]. Binding of the SH3 domain of p130cas to proline-rich region 1 in FAK was found to be required for prevention of apoptosis on fibronectin following serum withdrawal [78]. The FAK–p130Cas complex was found to activate c-Jun NH2-terminal kinase (JNK) via Ras/Rac1/Pak1/MAPK kinase 4 (MKK4) pathway. In a related study, extracellular-kinase activation (ERK) activation and CAS/Crk coupling are required to prevent apoptosis [79••]. Collectively, these studies demonstrate that cell–matrix interactions play an essential role in preventing apoptosis in normal cells and that this property is abrogated in transformed cells. It still remains to be determined how these pathways interact to regulate cell growth and survival and what role cytoskeletal elements plays in their function. The generation of signals induced by the interaction of cells with the ECM requires the maintenance of an intact cytoskeleton [81]. Furthermore, on the basis of results showing that adhesion-dependent signaling could be modulated by actomyosin contractility, it seems reasonable to predict that further analyses will reveal a pivotal role for the actin cytoskeleton in the regulation of survival signals generated by cell–ECM interactions.

Conclusions

Studies during the past few years have provided new clues regarding the relationship between the actin cytoskeleton and growth control. The direct interaction with cytoskeletal proteins during the activation of Rho and Rac — as described for hamartin/ezrin and Tiam1/ankyrin — most likely represents a paradigm for the action of other signaling molecules. These studies highlight the importance of the cytoskeleton in the spatial organization of signaling. It will be important to determine if the loss of stress fibers observed in transformed cell acts, in part by affecting the localization of signaling molecules. In addition, the actin cytoskeleton affects the organization and function of adhesive structures such as integrins and cadherins [14•,42], and alterations in the cytoskeleton can impinge on signaling pathways involving these. Cell–cell junctions play an important role in cell proliferation control and studies of the adenomatous polyposis coli highlight the role of junctional proteins involved in these structures [82]. Interestingly, overexpression of β-catenin was found to protect nontransformed epithelial cells from apoptosis, indicating that both integrin- and cadherin-containing structures play a role in the regulation of apoptosis [83].

Elucidating the mechanisms by which the interactions of cells with the ECM both generate and regulate downstream signals that promote cell growth and survival is critical to understanding cancer. Studies of the cytoskeleton will provide information regarding the possibility that these components might be potential targets for rational drug discovery for cancer. The regulation of myosin II and its relationship to adhesion-dependent signaling will require more studies to elucidate what downstream pathways are effected by activation or inhibition of force generation by myosin II motor molecules. In addition to the cytoplasmic events involving activation of myosin II and integrin signaling, it will be of interest to determine what genes are regulated in response to this type of signaling. Such studies will with no doubt provide important new insights into the genes involved in cell proliferation. With the advent of new microarray technologies, analyses of gene expression in various experimental models of cancer, as well as in human cancers, should provide important new insights in identifying specific cytoskeletal targets.

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